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ULTRASTRUCTURAL AND CYTOCHEMICAL EVALUATION OF THE  
CYTOTOXICITIES OF TRIM. (U) SOUTHEASTERN LOUISIANA UNIV  
HAMMOND DEPT OF BIOLOGICAL SCIENC. W M NORTON

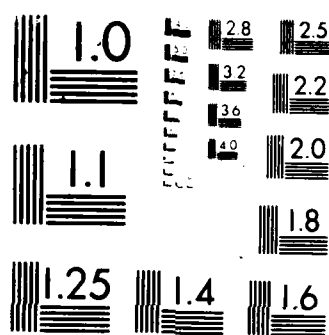
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ULTRASTRUCTURAL AND CYTOCHEMICAL EVALUATION OF THE CYTOTOXICITIES OF  
TRIMETHYLPENTANE ON RAT RENAL AND HEPATIC TISSUES

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## ABSTRACT

The primary objective of this investigation was to determine the acute cytopathologic effects of 2,3,4-trimethylpentane, a major constituent of gasoline, on renal and hepatic tissues of the mature male rat. Three groups of 9 Fischer 344 rats each were administered trimethylpentane by gavage twice weekly for 7, 14, or 28 days at a concentration of 1.5 ml/kg body weight. The tissues were fixed by perfusion, and subsequently processed for scanning and transmission electron microscopy. Within the glomerular complex there was a significant increase in the number of microvilli associated with various branches of podocytes. As compared to control glomerular complexes, experimental tissue demonstrated greater concentrations of injected cationized ferritin particles along the junction between the basal lamina and endothelium. Cells of the proximal convoluted tubule were characterized by membrane-bound, PAS positive hyaline droplets. At focal points along segments P<sub>1</sub> and P<sub>2</sub> of the proximal tubule intact epithelial cells dissociated from the basal lamina, underwent necrosis and subsequently collected along the length of the tubular lumen. The cellular debris concentrated at the corticomedullary junction. As monitored by horseradish peroxidase activity, the uptake of protein and subsequent formation of apical vesicles is greater among exposed epithelial cells of the proximal tubule than that of control tissue. There was no observable ultrastructural alterations among hepatocytes of exposed liver. The endoplasmic reticulum content appeared similar to control levels.

## Objectives

The primary objective of this investigation was to determine the acute cytopathologic effects of 2,3,4 - trimethylpentane on renal and hepatic tissues of the mature male rat. Selective cytochemical and electron microscopy related techniques were employed:

1. To characterize, ultrastructurally, renal lesions induced by trimethylpentane. The integrity and organizational relationships of cellular organelles were scrutinized for evidence of disruption.
2. To analyze by histochemical and electron microscopy related techniques the composition and structural arrangement of renal hyaline droplets whose formation were markedly induced by trimethylpentane.
3. To determine by means of horseradish peroxidase activity whether the protein uptake capabilities of cells comprising the proximal convoluted tubule were compromised by trimethylpentane.
4. To analyze, ultrastructurally, the organelle context of hepatocytes. Particular attention was directed to the smooth endoplasmic reticulum to determine whether trimethylpentane functions as an inducer of the detoxification system.
5. To determine by means of cationized ferritin tracers whether trimethylpentane altered the capability of the basal lamina to serve as a selective barrier to macromolecules.

## Materials and Methods

The hydrocarbon 2,3,4-trimethylpentane was obtained from Aldrich Chemical Company. Three groups of 9 Fischer-344 rats each were dosed by gavage twice weekly for 7, 14 or 28 days, respectively, at a concentration of 1.5 ml/kg body weight. Fifteen control animals were administered by gavage a comparable concentration of distilled water. Experimental and control animals were sacrificed at 7, 14 or 28 days subsequent to the initial exposure. Tissues selected for analysis by conventional electron microscopy were fixed by perfusion via the dorsal aorta at a hydrostatic pressure equal to 150 cm water.

The perfusate was 1% glutaraldehyde in 0.135M Sorensen's phosphate buffer (pH 7.2). After a perfusion period of 3 minutes, the kidneys and liver were excised and minced. Small segments of tissue were fixed for 1h by immersion in a fresh 0.135M Sorensen's phosphate buffered solution of 4% glutaraldehyde. The tissues were rinsed several times in 0.1M sodium cacodylate, and postfixed for 1h at 25°C in a 0.1M sodium cacodylate buffered solution of 1% osmium tetroxide. Subsequent to several rinses in fresh buffer, the tissues were dehydrated in a graded series of ethanol. The specimens were separated into 2 groups and further processed for

scanning and transmission electron microscopy, respectively. Samples selected for TEM were passed through 3 changes of propylene oxide and embedded in Epon 812. Thin-sections were cut by a Sorvall MT2 ultramicrotome, and stained with lead citrate and uranyl acetate. The sections were examined in a JEOL 100B transmission electron microscope at 60kV.

Tissues chosen for analysis by SEM were placed into small cylinders of parafilm which contained absolute ethanol. Both ends of each cylinder were crimped shut. The cylinder and its internal contents of ethanol and tissue were frozen in liquid nitrogen. The cylinder was placed on a flat metal block which had been precooled in liquid nitrogen. A precooled, single-edge razor blade was used to make a fracture through the cylinder and tissue. The fractured fragments were placed in absolute ethanol, and critical point dried using liquid CO<sub>2</sub> as described by Anderson (1951). The dried tissues were attached onto metallic stubs with silver conducting paint, and coated with a thin layer of gold. Specimens were observed in an ETEC Autoscan scanning electron microscope at 20kV.

Subsequent to perfusion, sections of kidney selected for a periodic acid-Schiff reaction were fixed for 24 hours in a neutral 10% formalin solution buffered with sodium acetate. Further processing of the tissues and the specific histochemical procedures were conducted according to the method described by Lillie and Fullmer (1976).

Experimental animals selected for the administration of cationized ferritin were injected with 0.5 ml. of the latter chemical at a concentration of 10 mg/ml. All injections were via the abdominal aorta. Subsequent to a perfusion period of 25 minutes, the kidneys were flushed with a rinse of buffer and perfused with a 2% glutaraldehyde solution for 3 minutes. The kidneys were excised, sectioned and placed in a cold solution of 4% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4). The tissues were post-fixed in 2% osmium tetroxide, rinsed in buffer and dehydrated in a graded series of ethanol. The samples were embedded in Epon 812, sectioned and observed on a JEOL 100B transmission electron microscope.

The animals to be investigated for protein uptake and distribution were injected intravenously with 8,000 units each of horseradish peroxidase. After 25 minutes the kidneys were perfused with a solution containing 2% glutaraldehyde. Subsequently the kidneys were excised, cut into small pieces and placed in 4% glutaraldehyde. The tissues were rinsed overnight in buffer and incubated for 30 minutes at 37°C in a saturated solution of 3, 3'-diaminobenzidine. The sections of tissue were rinsed and post-fixed for 60 minutes in 2% osmium tetroxide. The specimens were dehydrated in graded alcohols and embedded in Epon 812. The tissues were thin-sectioned and analyzed in a JEOL 100 B transmission electron microscope.



## Results

The various manifestations of hydrocarbon toxicity were evident in all experimental tissues examined. However, the extent and magnitude of cellular lesions as monitored by general observations of experimental tissue increased gradually as the exposure period progressed. All exposures were sublethal. The overall structural integrity of the glomerular complex was maintained throughout the duration of the study. The sole ultrastructural abnormality noted within the glomerular region was detected by scanning electron microscopy. As compared to control tissue (Fig. 1a) there was a significant increase in the number of microvilli (Table 1) associated with the primary, secondary and tertiary branches of podocytes (Fig. 1b). There was no discernible disruption of the endothelial lining or swelling of the basal lamina (Fig. 1c). The pedicels and their associated filtration slits appeared normal.

At focal sites along the length of segments  $P_1$  and  $P_2$  of the proximal convoluted tubule, epithelial cells displayed evidence of structural alteration. The most prominent feature of the affected cells was a marked increase in the number and size of pleomorphic hyaline droplets. The primary sites of proliferation consisted of cells comprising segment  $P_2$  of the proximal tubule, while the structures were evident to a lesser degree in segment  $P_1$ . The hyaline droplets stained positive for the periodic acid-Schiff reaction (Fig. 1d). The membrane-bound droplets were present as spherical bodies and irregularly shaped structures which possessed angular vertices (Fig. 2a). There appeared to be a sequential pattern to the formation of the specific shapes assumed by the droplets. Initially the spherical bodies were evident in affected cells, however, as the manifestations of toxicity intensified, the relatively large angular configurations appeared to be more prevalent. The hyaline droplets characterized by the angular shape displayed a crystalline core which demonstrated a periodicity of 3.57 nm (Fig. 2b). Such a precise pattern was not evident in the spherical droplets.

The effects of trimethylpentane toxicity among mitochondria of cells comprising the proximal tubule were evident in several structural anomalies. Many of the cells which contained hyaline droplets possessed mitochondria that appeared either swollen (Fig. 2c) or in a state of degradation as indicated by the presence of myelin figures. Also evident were mitochondria which contained an electron-dense matrix.

The apical canaliculi displayed no evidence of structural aberrations. Electron-dense coated vesicles were observed at the base of apical microvilli in all tissues examined. An analysis of segments  $P_1$  and  $P_2$  of the proximal tubules revealed distinct regions along the inner lumen where a loss of microvilli was evident (Fig. 2c). In several instances, the denudation of microvilli was noted among individual epithelial cells which dissociated from the basal lamina and collected in the lumen. There was little evidence of cellular fragmentation prior to

the dissociation. However, once within the lumen, cells appeared to undergo autolysis (Figs. 2c and 2d). Subsequently, the cellular debris tended to concentrate in the corticomedullary junction. The tubules were dilated focally and the epithelial lining was markedly attenuated (Fig. 3a).

To determine whether trimethylpentane affects protein uptake among cells of the proximal convoluted tubule, the horseradish peroxidase-diaminobenzidine technique was employed. The presence of proteins was monitored by an electron-dense product generated by the enzymatic alteration of 3,3'-diaminobenzidine by horseradish peroxidase. Reaction product was evident in apical vesicles associated with cells of proximal convoluted tubules of both experimental (Fig. 3b) and control animals. However, the greater concentration appeared to be present in vesicles of the experimental organisms. In addition, there was a concomitant increase in the number of apical vesicles.

Deposits of ferritin were evident along the junction between the basal lamina and endothelium of glomerular complexes of both control and experimental animals (Fig. 3c). However, particle distribution was not uniform. Ferritin was observed occasionally within the basal lamina and between the latter structure and podocytes. Ferritin was not detected in the urinary spaces. Cationized ferritin deposition appears to be greater in control as compared to experimental tissue.

There were no discernable ultrastructural changes noted among hepatocytes of exposed liver (Fig. 3d). The orientation and distribution of cellular organelles was similar to that of control tissue. The concentration of endoplasmic reticulum appeared to be consistent throughout the exposure period. Nuclear features such as the extent of hetero and euchromatin and nucleolar organization were similar to those of control hepatocytes. There was no evidence of disruption or alteration of the biliary system or cells associated with the sinusoids.

TABLE 1

The number of glomerular microvilli per unit area. Values were obtained from 6 rats for each of the specific time points and represented an analysis of 10 random sites per organ.

Control	<u>Exposure Period in Days</u>		
	7	14	28
6.7 $\pm$ 2.7*	10.5 $\pm$ 3.2*	16.5 $\pm$ 1.9*+	24.3 $\pm$ 5.4*+

\* Mean and standard error

+ Significant at the 0.05 confidence level

## Discussion

The determination that exposure of gasoline vapors to male rats results in nephrotoxicity and carcinogenicity (MacFarland, 1984) suggests a potential risk to the general population exists. However, an interesting facet of this issue and one which may impact on risk assessments concerns the inability of hydrocarbons associated with gasoline to induce cellular lesions or neoplastic changes in other organs and female rats. There is an apparent relationship between trimethylpentane toxicity and the proliferation of hyaline droplets among cells of the proximal tubule. A variety of petroleum-based and synthetic hydrocarbons have proven to be initiators of nephrotoxicity and, more specifically, inducers of hyaline droplet formation (Bruner, 1984; Halder et al., 1984). Investigators have speculated that accentuated hyaline droplet production may be dependent upon a sex related, low molecular weight protein, alpha 2 u globulin (MW 26,400) (Bruner, 1984; Irwin et al., 1971). The protein is synthesized by the liver of male rats at puberty under the influence of testosterone. The globulin is readily filtered through glomeruli of the kidney, and represents the primary urinary protein of male rats (Roy, 1973; Roy and Neuthaus, 1966; 1976). There is no indication that the protein is synthesized by female rats.

Hyaline droplets represent structures which usually result from absorption of proteins from the glomerular filtrate. Subsequently, the proteins concentrate in large apical vesicles which are transformed into lysosomes (Kretschmer and Bernstein, 1974). Occasionally, the hyaline droplets represent an expression of disturbed cellular metabolism. For example, droplets develop in cells of collecting tubules during potassium deficiency, and are a characteristic feature of malignant nephrosclerosis (France, et al., 1974; Churg et al., 1980).

The results of this study indicate that there is not a detectable increase in the rough endoplasmic reticulum content or secretory vesicles of hepatocytes exposed to trimethylpentane, which indicates that the biosynthesis of the globulin may not be enhanced by hydrocarbons. However, the increase in apical vesicles and electron-dense material within those structures indicates that epithelial cells of the proximal convoluted tubule are absorbing more protein than comparable cells of control tissue.

Bruner (1984) has postulated that among male rats exposed to selective hydrocarbons, the 2 u globulin represents the major constituent of hyaline droplets, and the biochemical and cytological consequences of its excessive accumulation in proximal tubular cells relate to the manifestations of cytotoxicity. The accentuated concentration of droplets may reflect an inability to degrade enzymatically what would normally be an innocuous protein, once uptake has occurred.

The positive periodic acid-Schiff reaction demonstrated by hyaline droplets present in epithelial cells of kidney exposed to trimethylpentane indicates the presence of a carbohydrate moiety. Results of the PAS reaction tend to support the supposition that the structures possess hyaline since previous studies have verified the presence of carbohydrates in hyaline droplets (Churg et al., 1980).

Ultrastructural observations indicate that as the hyaline droplet content of tubular cells increases during progressive exposure, there is a concomitant change in the three-dimensional configuration of the structures from spherical to angular bodies. The transformation in shape may be correlated with a chemical or physical alteration of the internal contents, since analysis of the spherical bodies reveal no discernible substructure, whereas the angular droplets are characterized by a periodicity. Such a crystalline formation indicates that a proteinaceous complex, conceivably 2 u globulin, concentrates in the hyaline droplets as a result of exposure to trimethylpentane. The apparent relationship between elevated levels of hyaline droplets and the dissociation of epithelial cells from the basal lamina represents a phenomenon which currently is unresolved. There appears to be no degradation of the basal lamina prior to the dissociation, and the plasma membrane maintains its integrity until the cell settles in the lumen.

Cells aligning the compromised segments of proximal tubules display no evidence of the classical manifestations of regeneration such as an abundance of free polysomes, a paucity of other organelles and a large lobed nucleus with diffuse chromatin. However, during a period of recovery cellular regeneration would seem possible since the basal lamina remains intact structurally, and significant numbers of cells in the affected segments appear to be unaltered by trimethylpentane. Renal tissue of rats exposed to hydrocarbons has demonstrated an ability to recover physiologically from toxic insults induced by the compounds. D'Addario et al. (1985) report that rats continuously exposed to jet fuel vapor for 90 days experience a significant reduction in the urine concentrating ability of the kidneys. All biochemical indicators return to control levels following a 9 week recovery period.

Whereas the consistent proliferation of hyaline droplets indicates an indirect effect of trimethylpentane toxicity, the transformations detected among mitochondria regarding swelling and degradation intimate direct manifestations of hydrocarbon exposure. Comparable toxic effects on mitochondria have been reported for hepatocytes of fat-head minnows exposed to aromatic hydrocarbons (Norton et al., 1985). The extent of mitochondrial dysfunction among tubular cells has not been determined. However, the organelles display structural changes which indicate both reversible and irreversible damage.

There is no evidence of structural insult to the basal lamina associated with the glomerular complex. However, the increase in ferritin particles within and along the basal lamina indicates the possibility of a change in the net overall charge of the structure. The authors are not aware of any reports concerning the modifications of surface charge of the basal lamina.

Epithelial cells of the proximal tubule which contain prominent hyaline droplets undergo a marked reduction in the number of apical microvilli. Such an effect is not unexpected since microvilli are highly sensitive to injury and frequently respond by becoming ballooned or reduced in number (Churg et al., 1980). However an interesting

consequence of trimethylpentane exposure on the glomerular complex concerns the significant increase in number of microvilli associated with podocytes. The authors are not cognizant of other conditions which may induce such a selective effect. Hydrocarbons appear to alter cilia in just the opposite manner. Epithelial cells of the nasal mucosa of fat-head minnows exposed to aromatic hydrocarbons are characterized by a loss of cilia (Norton et al., 1985).

In summary, this investigation represents an initial attempt to analyze by scanning and transmission electron microscopy the ultrastructural effects of an acute exposure of trimethylpentane to renal and hepatic tissues of the sexually mature rat. Several pertinent manifestations of toxicity are evident, including the dissociation and subsequent necrosis of epithelial cells from the basal lamina of specific segments along the proximal convoluted tubules. The cytotoxicity of affected epithelial cells appears to be associated with the proliferation of PAS positive hyaline droplets which consist of crystalline material as demonstrated by an internal periodicity. Specific cytochemical analysis indicates an enhanced uptake of protein by cells of the proximal tubule. Future investigations should be designed to determine the biochemical nature of the hyaline droplets, and analyze the cause and effect relationship, if any, between the degradation of proximal tubular cells during acute exposure and the carcinogenicity associated with chronic exposure.

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### **Publications Relating to Research Endeavors**

The following articles pertain to the research activities described in the final report.

Norton, William and David Mattie. 1986. The cytotoxic effects of trimethylpentane on rat renal tissue. International Journal of Scanning Electron Microscopy. Accepted for publication.

Norton, William and David Mattie. The influence of trimethylpentane on protein uptake by cells of the rat proximal convoluted tubule. Journal of Histochemistry and Cytochemistry. In preparation.

### **Papers Presented at Scientific Meeting**

Scanning Electron Microscopy meeting. Title of presentation:  
"Manifestations of Trimethylpentane Exposure on Rat Renal Tissue".  
New Orleans, Louisiana, 1986.





Figure 1a. Surface of glomerular complex with orderly arranged pedicels (PE) and tertiary branches (TB) of podocytes. Note sparsity of microvilli (arrow). Control tissue. X 11,000.

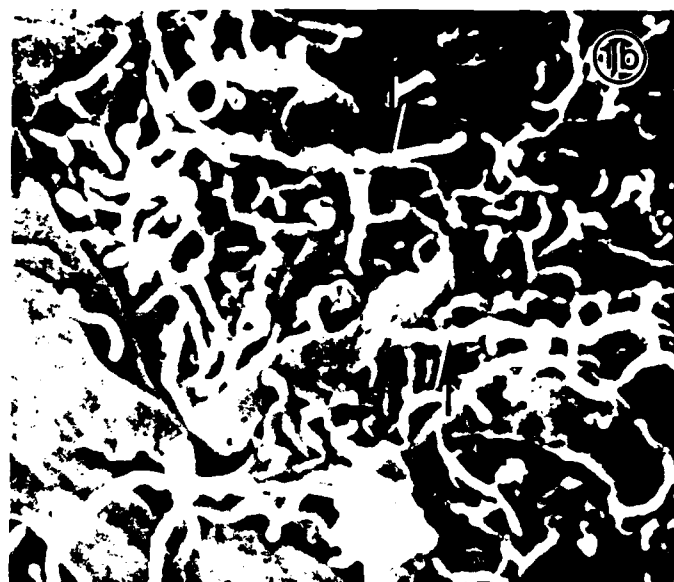


Figure 1b. Proliferation of microvilli (arrows) along the length of glomerular complex. Experimental tissue - 14 days of exposure. X 14,000.

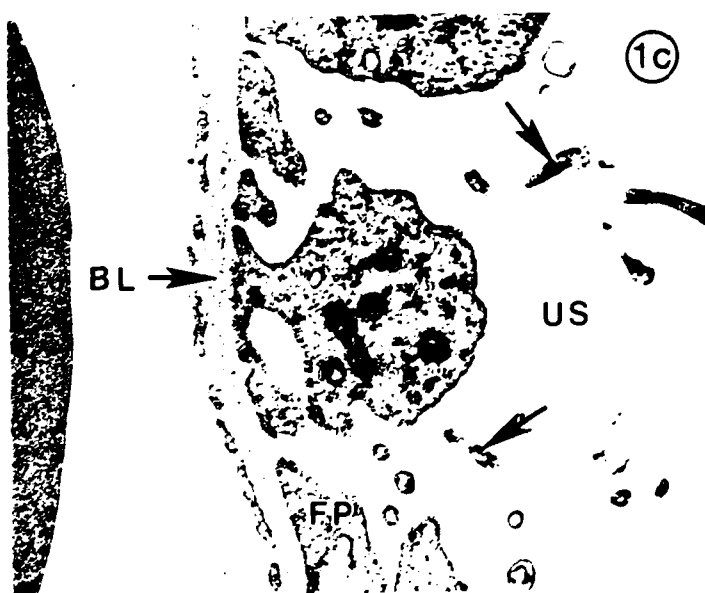


Figure 1c. Multiple microvilli (arrows) extend into urinary space (US). Basal lamina (BL) and foot processes (FP) of pedicels appear normal. Experimental tissue - 14 days of exposure. X 45,000.

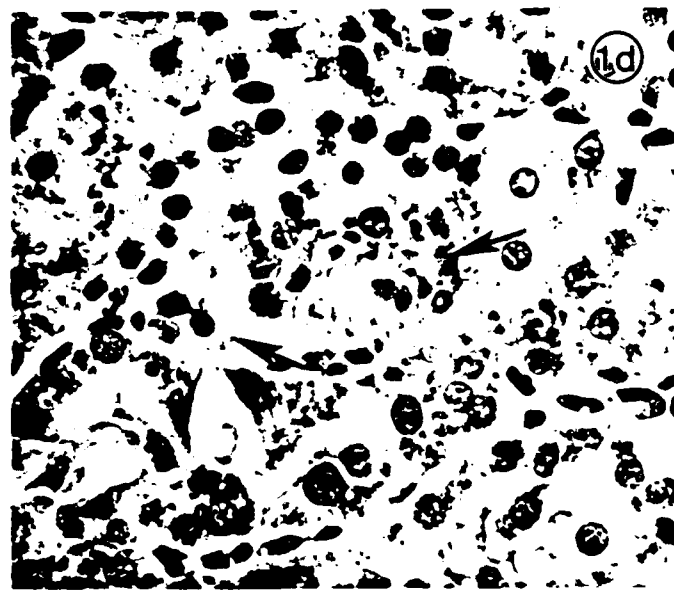


Figure 1d. Bright field photomicrograph reveals the presence of PAS positive hyaline droplets (arrows). Proximal tubule associated with the pleomorphic droplets appears disrupted. Experimental tissue - 7 days of exposure. X 1,500.



Figure 2a. Proximal tubular cell contains several hyaline droplets (HD), one of which is characterized by angular vertices (arrows). Experimental tissue - 7 days of exposure. X 16,000.

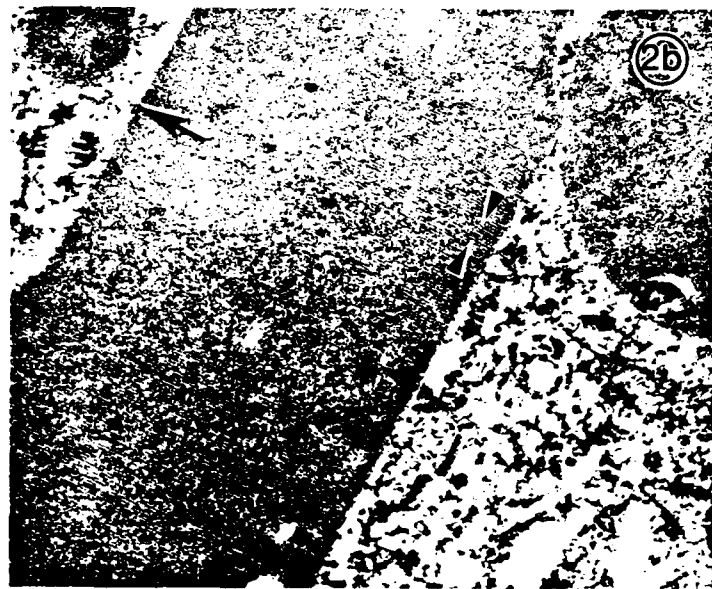


Figure 2b. Hyaline droplet is membrane-bound (arrow) and demonstrates an internal periodicity (arrowheads). Experimental tissue - 14 days of exposure. X 80,000.

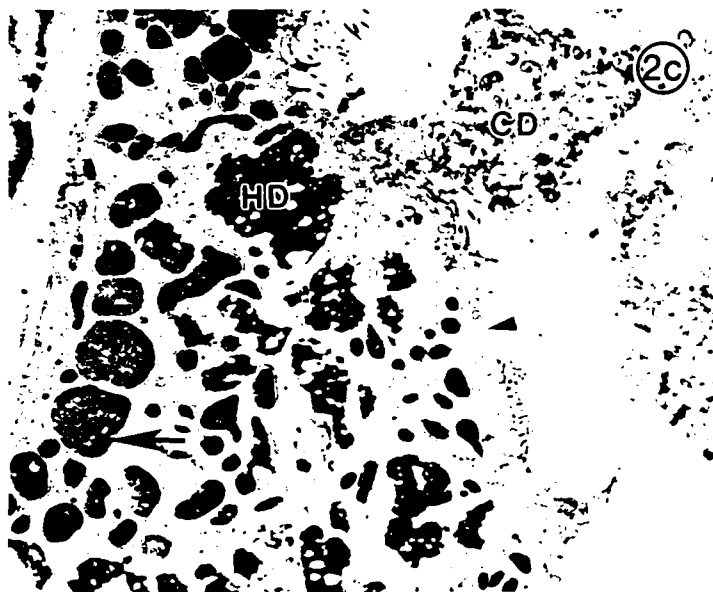


Figure 2c. Proximal tubular cell is characterized by swollen mitochondria (arrow), hyaline droplets (HD) and reduced concentration of microvilli (arrowhead). Note cellular debris (CD) in lumen. Experimental tissue - 14 days of exposure. X 13,000.



Figure 2d. Hyaline droplets (arrows) and microvilli (arrowhead) have been released from fragmented cell into lumen of proximal tubule. Experimental tissue - 14 days of exposure. X 9,000.

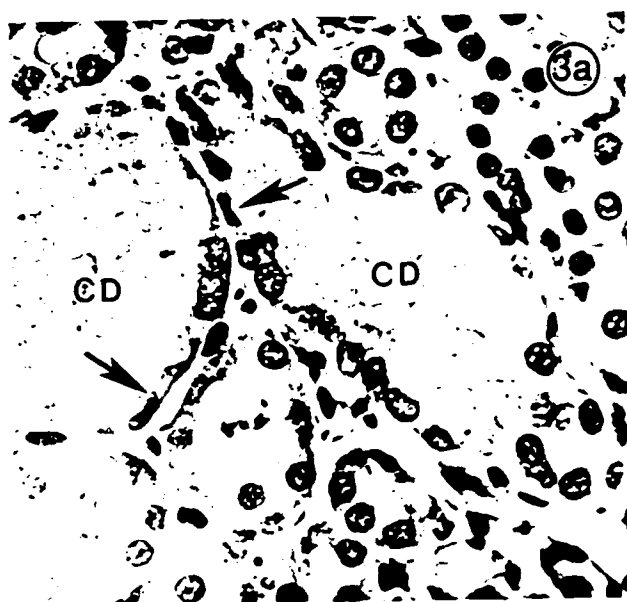


Figure 3a. Substantial accumulation of cellular debris (CD) in the general region of the corticomedullary junction results in dilation of the tubules and attenuation of the epithelial lining (arrows). Experimental tissue - 28 days of exposure. X 1,500.



Figure 3b. Apical region of epithelial cell from proximal convoluted tubule depicts numerous vesicles (arrow) which contain various amounts of an electron-dense material which forms as the result of reactions involving horseradish peroxidase. Also evident is a hyaline droplet (HD). Experimental tissue - 7 days of exposure. X 20,000.



Figure 3c. Section through glomerular complex illustrates basal lamina (BL), foot processes of podocytes (PO) and endothelium (arrowhead). Note sparsity of ferritin particles (arrow). Experimental tissue - 14 days of exposure. X 35,000.

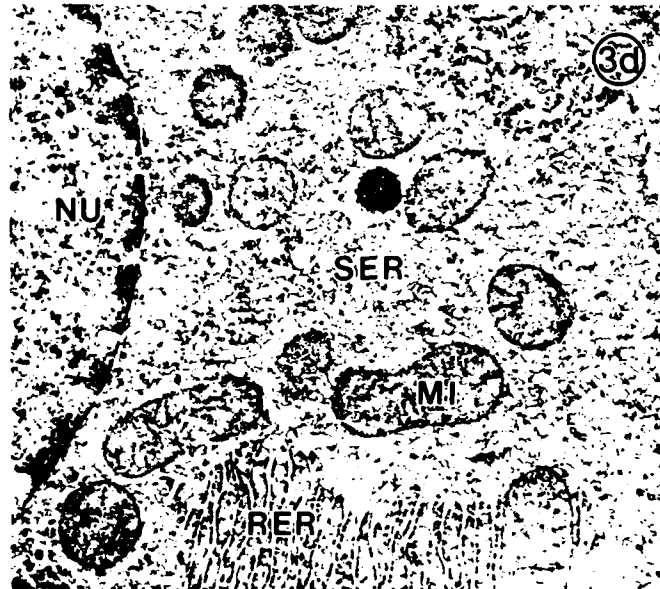


Figure 3d. Normal appearing internal organelles of hepatocytes include parallel arrays of rough endoplasmic reticulum (RER), elongated mitochondria (MI) and concentrated clusters of smooth endoplasmic reticulum (SER) adjacent to a spherical nucleus (NU). Experimental tissue - 28 days of exposure. X 9,000.

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